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(56) Documents Cited
Biotechnology Techniques Vol.8 (7) 1994. Kimura K & Kobayashi K. pages 473-478 BioTechniques Vol.11 (1) 1991. Cole K D. pages 18-24 Appl. Biochem & Biotech. Vol.26 (3) 1990. Sturesson S et.al. pages 281-295

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(54) Abstract Title
Separation of nucleic acids in a two-phase system

(57) A method of extracting nucleic acids, avoiding the use of guanidinum salts, is described. The reagents comprise water, a water miscible organic solvent, preferably a short chain alcohol, and a partitioning agent to cause biphasic separation yielding the nucleic acid in the bottom layer aqueous layer. The partitioning agent is preferably an inorganic salt such as a phosphate, sulphate or carbonate which is mixed initially with the nucleic acid containing material before the addition of the solvent, preferably ethanol. The mixture is preferably subject to light centrifugation, for example 5000g for one to five minutes, to facilitate separation. Preferably, prior to treatment the nucleic acid material is heated to denature proteins.

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Figure 1

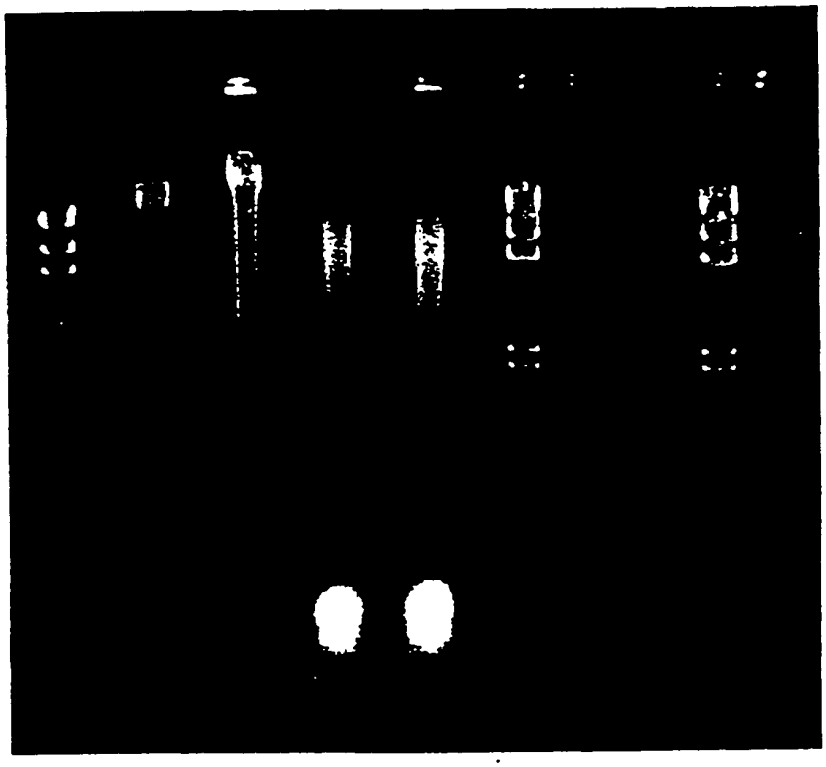


Figure 2

M 1 2 3 4 5

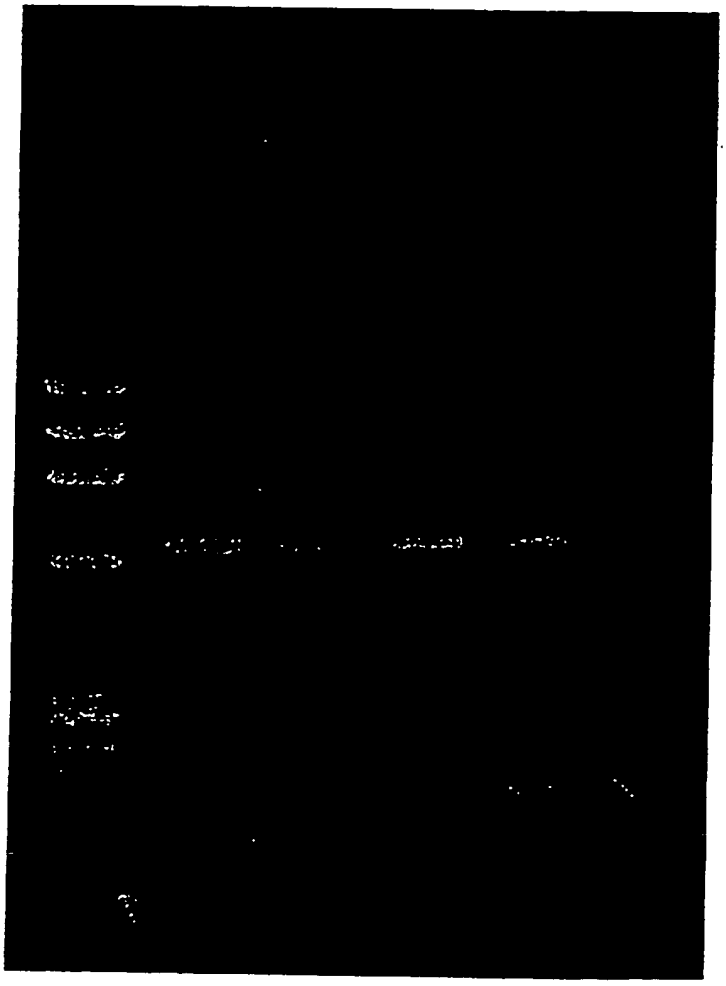


Figure 3

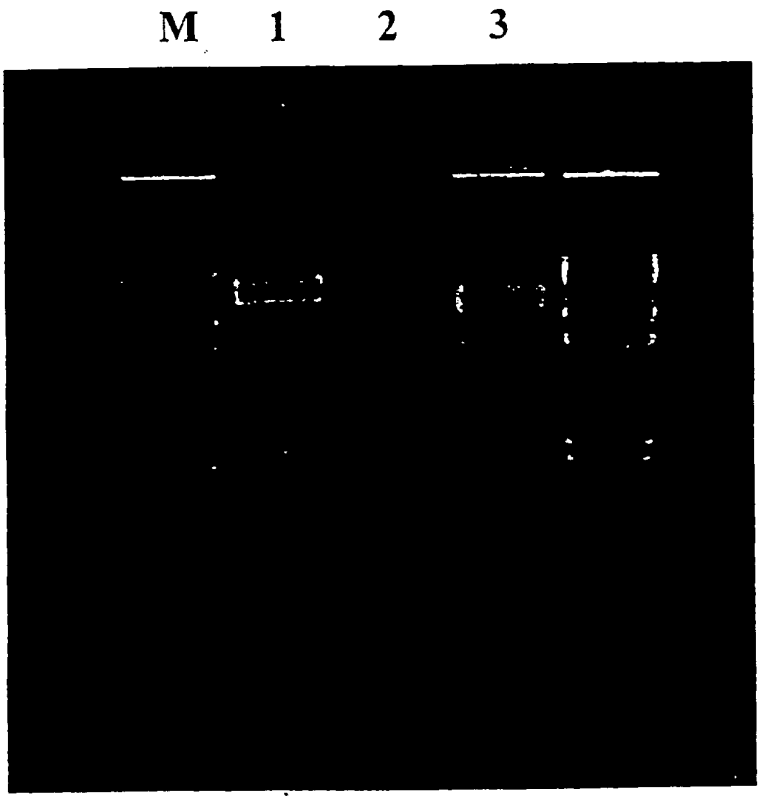
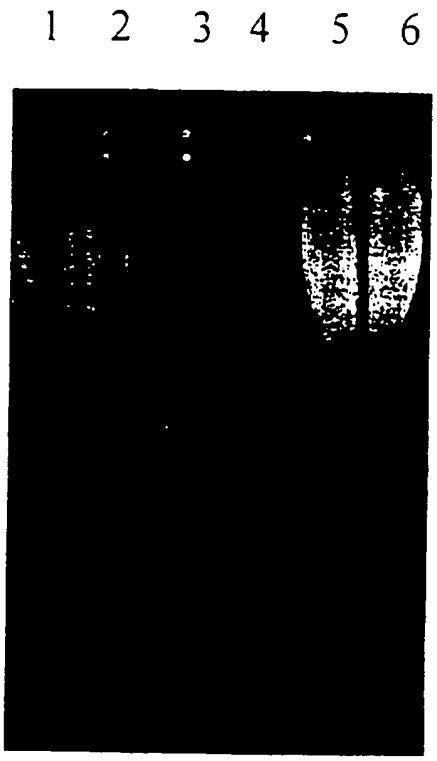


Figure 4



METHOD OF NUCLEIC ACID EXTRACTION

Field of the Invention

The present invention relates to a method of extraction of nucleic acids from nucleic acid-containing biochemical material.

Background to the Invention

The vast and proliferating global biotechnology industry relies heavily upon techniques to extract nucleic acids from biochemical materials before any of the myriad DNA recombination techniques may be performed or further research carried out on the nucleic acid materials. Most commonly, there is a need to isolate DNA from enzymes and other proteins. Conversely, the protein may be the desired end product of the extraction and require removal of the DNA. A host of molecular biology techniques such as cloning, restriction analysis or the polymerase chain reaction (PCR); (Saiki et al (1985) *Science* **230**, 1350) require DNA which is substantially free of contamination (where contaminants are defined as those substances that would inhibit or substantially alter the results of the molecular biology process in question) in order to be carried out. Contamination might, for example, be by protein, lipid or carbohydrate, but of these protein contamination is the primary problem to be addressed for most procedures.

The principle problem with obtaining nucleic acids of interest free from contaminating substances is greatly complicated by the complexity of the systems in which they are found. It is necessary to separate these contaminants as efficiently as possible without untowardly damaging or degrading the nucleic acids in question. The complexity of biochemical materials, being materials of biological nature or origin, is illustrated by the following examples from where nucleic acids

are often isolated: bacteria (micro-organisms) yeasts, plants, human (or other animal) samples of blood, semen, tissue, faeces or urine. Essentially, nucleic acids and in particular DNA may be extracted from a very wide range of biochemical samples, possibly even in a state of fossilisation, as long as the required nucleic acids are present.

Methods for the extraction and isolation of nucleic acids are widely documented, for example in text books such as Current Protocols in Molecular Biology published by W Wiley- Inter Science, New York (1993). DNA isolation is generally achieved by cell lysis, DNA solubilization, followed by a chemical or enzymatic process designed to reduce contaminants to levels too low to interfere with subsequent experimentation or use. RNA isolation requires cell lysis and solubilization procedures, with concomitant ribonuclease inhibition and separation from (contaminating) DNA.

There are a variety of DNA extraction methods. One version requires the use of phenol/chloroform as an extracting agent or phenol/chloroform/isoamyl alcohol followed by precipitation by ethanol. This method has significant drawbacks, as several steps are involved in the process, hence the procedure takes a significant amount of time. Furthermore, there are dangers associated with the use of phenol and chloroform. Phenol causes severe burns or blindness if contact is made with the skin or eyes, respectively. Chloroform has been recognised as possibly having irreversible effects on human health, as well as being volatile, toxic and flammable. Use of both chemicals require careful handling in a fume hood and protective equipment for their use. From a biochemical point of view, phenol oxidation produces products that can damage nucleic acids (which hence cannot be

stored or left in the medium), and hence the phenol needs to be prepared (distilled) fresh before use. Furthermore, ethanol (or isopropanol) is required to precipitate DNA from the phenol/chloroform mixture and remove those chemicals, as well as nucleoside triphosphates and small oligonucleotides from the isolated nucleic acid (DNA). Cesium chloride centrifugation can be used to isolate DNA, however, this requires the use of an ultra-centrifuge capable of centrifuging samples between 150,000 to over 525,000g for several hours, a costly and lengthy procedure. This latter procedure is only useful for small samples of DNA.

Preparations using cetyltrimethylammonium bromide (CTAB) exist for DNA extraction. However, CTAB is suspected to affect reproductive organs, as well as being an irritant, harmful, and an environmental hazard, being particularly dangerous to aquatic life. Another method requires the use of glass/silicates, such as glass beads or diatomaceous earth. The method requires a chaotropic binding agent, although alternative approaches, such as the use of hydrophilic coatings on silica to bind proteinacious material leaving the DNA behind (US patent application number 4, 923, 978) or using ethanol as a binding agent (European patent application number 0512767 A1) have been shown. Other solid support methodologies, including magnetic silica extraction, work on the same principle followed by magnetic separation of the particles after adsorption of the DNA to the silica using a magnet (PCT WO 83/03920).

RNA extraction methods can essentially be categorised into three basic methods:

(1) extraction using phenol/chloroform;

(2) extraction using protease degradation with strong detergents, such as sodium dodecyl sulphate (SDS); or

(3) extraction using strong salts.

The problems of using phenol and/or chloroform have already been highlighted. The detergents such as SDS must be removed in order to use the RNA at a later stage. There is also evidence to suggest that SDS itself may have effects on the reproductive system as well as being a known irritant. Finally, salts themselves do not produce RNA free of proteins. Phenol is still used in conjunction with salts to remove protein (Chomczynski and Sacchi, (1987) Anal. Biochem. **162**, 156). Alternatively, a centrifugation step can be employed. These methods are time consuming, and where phenol or SDS is used, potentially dangerous.

The use and construction of aqueous biphasic systems has been well documented for some time (*Chromatogr.B* (1996) **680**) but not in the context of nucleic acid extraction. Generally, they are constructed using two different polymers, such as polyethylene glycol (PEG) and dextran, or a single polymer (such as PEG) and salt solutions. Biphasic solutions using organic solvents have not been widely studied. This is probably because the hydrophilic solvents (in which some proteins or nucleic acids have appreciable solubility), such as dimethyl sulphoxide (DMSO) or methanol cannot form a biphasic system with water in the first place, as they are infinitely miscible with water in all proportions. Water-immiscible organic solvents can form two phases with water, however, they have such limited solubilities for proteins or nucleic acids that they are essentially not useful for separation of these types of compounds. PEG-salt biphasic aqueous systems has been used to separate nucleic acids from proteins (Cole, KD, 1991) *Biotechniques* **11**, 18). However, some of these systems still require the addition of chaotropic agents, and can be pH sensitive so that yields of nucleic acids may be lower than desired.

Summary of the Invention

According to the present invention there is provided a method of nucleic acid extraction from nucleic acid containing biochemical material avoiding the use of guanidinium salts, which method comprises the use of a biphasic system made up of

(i) a water miscible organic solvent and

(ii) water, in combination with a partitioning agent.

The two solvents, the water miscible organic solvent and water, are normally 100% miscible. However when partitioning agents comprising inorganic salts such as potassium phosphate or certain specialised polymers are added to the water component a biphasic system ensues.

Where the primary objective of the method is to isolate the nucleic acid from proteins in the biochemical material the applicant has found surprisingly that by far and away the best form of water miscible organic solvent is a short chain alcohol or mixture thereof. Particularly preferred short chain alcohols are ethanol and propanol.

For the partitioning agent the most effective appear to be potassium phosphate (mono-,dibasic or tribasic). Also preferred are magnesium sulphate, disodium carbonate and disodium sulphate or any mixture of these inorganic salts.

Where the method of nucleic acid extraction is not specifically for separating the nucleic acid from proteins alternative water miscible organic solvents such as acetonitrile, tetrahydrofuran and acetone are usable.

Depending on which salt and organic solvent combinations are used, the concentration of salt necessary to create the biphasic system varies but this may be readily determined by routine experimentation. In the case of potassium phosphate,

the value varies between about 10% and 100% saturation, when equal volumes of ethanol and the salt solution are added. Other salts can be used to form a biphasic ethanol aqueous system, including, for example, magnesium sulphate (20%-60% saturation), disodium carbonate (70% saturation), and disodium sulphate (70%-75% saturation).

Where an organic solvent such as acetonitrile and tetrahydrofuran is used the appropriate inorganic salt is suitably calcium chloride or potassium chloride. For acetone the most appropriate salt is calcium chloride. These salts would normally be added in equal volumes to the salt solution.

In order for extraction of nucleic acids to take place the biochemical material is suitably initially made up as an aqueous solution of either whole cells or cell free extract together with aqueous solution of the inorganic salt or other partitioning agent. To this an equal volume (or volume resulting in a biphasic system) of ethanol or other water miscible organic solvent is added. The solution is then agitated to ensure good mixing after which it is left to stand on a bench in order for the two phases to settle out.

Preferably the method further comprises a centrifugation step (1 to 5 minutes at about 5000g) if the solution is very viscous or contains much protein and/or DNA, thus aiding in the separation of the two phases. Such a centrifugation step is, however, much briefer and less demanding than required for prior art procedures.

Generally, the resulting bottom (aqueous) phase including the interface will contain the nucleic acids, while the top (organic phase) will contain the vast majority of the protein (depending on which biphasic system is being used).

The pH can be altered according to requirements, although this too can have an effect on separations. The method can be used to extract the nucleic acids for further use, or for their removal from the protein or other desired components of the biochemical material.

A mild heat pre-treatment of the biochemical material to denature proteins (40°C to 90°C, for example,) before carrying out the extraction with the biphasic system may further enhance the efficiency of extraction.

Brief Description of the Figures

The present invention will now be more particularly described by way of example with reference to the accompanying Figures and five specific experimental examples.

The accompanying Figures 1 to 5 each represent a respective photograph of a respective DNA aqueous gel electrophoresis comprising the results of the five experimental examples.

EXAMPLE 1 Prokaryotic (*Escherichia coli*) DNA extraction/removal

A starter *Escherichia coli* (*E. coli*) culture (ATCC11303, strain B from Sigma/Aldrich) was grown in 100 ml 2 x YT media (ampicillin 100 µg/ml) and incubated overnight at 37°C. 8 ml of overnight starter culture was added to 400 ml broth (2 x YT media; ampicillin 100 µg/ml) and grown at 37°C for 16 h. The cells were harvested and spun down (4000g; 15 minutes), and resuspended in 12 ml wash buffer (Tris-HCl; 50 mM; pH 7.6; 1mM EDTA). The washing step was repeated twice, after which the cells were resuspended in 24.0 ml Tris lysis buffer (20 mM; pH 8.0, 0.1 mM EDTA). 80 µL of lysozyme (50 mg/ml) was added, and incubated for 15 min at room temperature. The solution was then incubated for 20 min at 75°C, put into centrifuge tubes and spun at 18,000g for 25 minutes (crude extract). Saturated potassium phosphate was added to a final concentration of 20%(v/v) saturation, and an equal volume of ethanol added. The resultant biphasic mixture was left to separate (although centrifugation can be used to speed up the process). The original crude extract as well as both layers from the biphasic system were removed, desalted, and samples run on a 1% agarose gel at 60 volts for 2 hours, stained with ethidium bromide, and visualised under UV transillumination.

The results are given in Figure 1, showing the original crude extract (lane 1), and the top (organic) phase (lane 2), the bottom (aqueous) phase from the extraction (lane 3). It is clear that all the nucleic acid is absent from the organic (top) phase, but present in the bottom (aqueous) phase.

EXAMPLE 2 Eukaryotic (*Saccharomyces cervisiae*) DNA extraction/removal

Same as EXAMPLE 1, but that Bakers yeast was used (*Saccharomyces cerevisiae*; Type II from Sigma/Aldrich).

The results are given in Figure 1, showing the original crude extract (lane 4), and the top (organic) phase (lane 5), the bottom (aqueous) phase from the extraction (lane 6). It is clear that all the nucleic acid is absent from the organic (top) phase, but present in the bottom (aqueous) phase.

EXAMPLE 3 Human blood DNA extraction/removal

5 ml frozen blood was diluted with 15 ml deionised and distilled water, and spun at 500 g for 2 minutes to isolate white blood cells/nuclei, and resuspended in 1 ml water. Saturated potassium phosphate at pH 7.0 was added to a final concentration of 20%(v/v) saturation, and an equal volume of ethanol added. The resultant biphasic mixture was left to separate (although centrifugation can be used to speed up the process). Both layers from the biphasic system were removed, desalted, and samples run on a 1% agarose gel at 60 volts for 2 hours, stained with ethidium bromide, and visualised under UV transillumination. The results are given in Figure 1, showing the bottom phase (lane 7), and the top (organic) phase from the extraction (lane 8). It is clear that all the nucleic acid is absent from the organic (top) phase, but present in the bottom (aqueous) phase. The isolated DNA with a 260/280 nm ratio of 1.8 (sufficiently pure for nucleic acid amplification) was successfully used for PCR of a ß-actin fragment 653 base-pairs long (Figure 2, lanes 1-4).

EXAMPLE 4 Recombinant bacterial (*E. coli* with *Thermus aquaticus* DNA polymerase gene) DNA extraction/removal

Same as EXAMPLE 1, except that the strain used was *E. coli* (containing the pTAQ construct; DSBI-alpha-1). This culture produces the *Thermus aquaticus* DNA polymerase (Taq; Lawyer et al. (1993) *PCR Methods and Applications* 2, 275), which can be used in PCR. The main culture was grown to an OD (600 nm) of 0.3 (approximately 1.5-1.75h), whereupon 480 µL IPTG (20% w/v, or 0.84 M) was added. It was grown at 37°C for 16h.

The results are given in Figure3, showing the original crude extract (lane 1), and the top (organic) phase (lane 2), the bottom (aqueous) phase from the extraction (lane 3). It is clear that all the nucleic acid is absent from the organic (top) phase, but present in the bottom (aqueous) phase.

Both top (organic) and bottom (aqueous) layers were separated, desalted, and were assayed for Taq activity, using PCR, and the results compared to PCR carried out using the original crude extract. 100% of Taq activity was preserved, and present in the top (organic phase), clear of contaminating nucleic acids. 4-12% gradient sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS PAGE) showed no detectable protein to be present in the bottom phase. However, the top phase showed a protein matching the molecular weight of Taq (94 kd), as well as other minor contaminating bands.

EXAMPLE 5 Recombinant bacterial (*E. coli* with *Thermus aquaticus* DNA polymerase gene) DNA extraction/removal using an iso-propanol and dipotassium hydrogen phosphate biphasic system.

Same as EXAMPLE 4. except equal volumes of iso-propanol and 10% saturated dipotassium hydrogen phosphate were used to form a stable biphasic system.

The results are given in Figure 4, showing the original crude extract (lanes 1, 2), and the top (organic) phase (lanes 3, 4), the bottom (aqueous) phase from the extraction (lanes 5, 6). It is clear that all the nucleic acid is absent from the organic (top) phase, but present in the bottom (aqueous) phase.

Both top (organic) and bottom (aqueous) layers were separated, desalted, and were assayed for Taq activity, using PCR, and the results compared to PCR carried out using the original crude extract. 100% of Taq activity was preserved, and present in the top (organic phase), clear of contaminating nucleic acids. 4-12% gradient sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS PAGE) showed no detectable protein to be present in the bottom phase. However, the top phase showed a protein matching the molecular weight of Taq (94 kd), as well as other minor contaminating bands.

Legend to Figure 1; DNA agarose gel (1%)

M Marker (Lambda *Hind* III; sizes from top to bottom in base pairs: 23130, 9416, 6557, 4361, 2322, 2027, 564 and 125)

1 Crude *E. coli* extract

2 Upper (organic) layer after biphasic extraction of crude *E. coli* extract

3 Lower (aqueous) layer/interface after extraction of crude *E. coli* extract

4 Crude yeast extract

5 Upper (organic) layer after biphasic extraction of crude yeast extract

6 Lower (aqueous) layer/interface after extraction of crude yeast extract

7 Lower (aqueous) layer after biphasic extraction of white cells/nuclei extracted from 5 ml human blood

8 Upper (organic) layer/interface after extraction of white cells/nuclei extracted from 5 ml human blood

Legend to Figure 2; DNA agarose gel (2%)

M Marker (ØX174/Hae III, sizes from top to bottom in base pairs: 1353, 1078, 872, 603, 310, 281, 271, 234, 194, 118, 72).

1 β-actin PCR using 400ng DNA

2 β-actin PCR using 200ng DNA

3 β-actin PCR using 100ng DNA

4 β-actin PCR using 50ng DNA

5 Negative control, no added DNA

Legend to Figure 3; DNA agarose gel (1%)

M Marker (Lambda *Hind* III; for sizes see Figure 1)

1 Crude recombinant *E. coli* extract

2 Upper (organic) layer after biphasic extraction of crude *E. coli* extract

3 Lower (aqueous) layer after biphasic extraction of crude *E. coli* extract

Legend to Figure 4; DNA agarose gel (2%)

M Marker (Lambda *Hind* III; for sizes see Figure 1)

1, 2 Crude recombinant *E. coli* extract

3, 4 Upper (organic) layer after biphasic extraction of crude *E. coli* extract

5, 6 Lower (aqueous) layer after biphasic extraction of crude *E. coli* extract

CLAIMS:

1. A method of nucleic acid extraction from nucleic acid containing biochemical material avoiding the use of guanidinium salts, which method comprises the use of a biphasic system made up of (i) a water miscible organic solvent and (ii) water, in combination with a partitioning agent.

2. A method as claimed in Claim 1, wherein the water miscible organic solvent comprises a short chain alcohol.

3. A method as claimed in Claim 1 or Claim 2, wherein the partitioning agent is an inorganic salt.

4. A method as claimed in Claim 3, wherein the inorganic salt is selected from the group consisting of potassium phosphate, (mono-, dibasic or tribasic), magnesium sulphate, disodium carbonate or disodium sulphate or any mixture of these.

5. A method as claimed in any preceding claim, wherein the method comprises the steps of taking the biochemical material from which the nucleic acid is to be extracted and mixing it with the partitioning agent, one or both of the biochemical material and partitioning agent being an aqueous solution, and then adding the water miscible organic solvent; and agitating to ensure good mixing and leaving to stand for the two phases to settle out.

6. A method as claimed in Claim 5, wherein the method comprises the further step of physically separating the lower aqueous phase from the upper organic phase of the biphasic system.

7. A method as claimed in any preceding claim, wherein the biphasic system is subjected to centrifugation.

8. A method as claimed in claim 7, wherein the centrifugation is carried out for one to five minutes at or around 5000g.

9. A method as claimed in any preceding claim, which method further comprises the step of pre-treating the biochemical material with heat sufficient to denature proteins in the biochemical material.

10. A method as claimed in claim 9, wherein the heat treatment is carried out at a temperature of between 40 and 90°C.

11. A kit for use in the method of any preceding claim, wherein the kit comprises a water miscible organic solvent in a first vessel and partitioning agent in a second vessel and instructions for use of the two to set up a biphasic system for extraction of nucleic acid from nucleic acid containing biochemical material.

Amendments to the claims have been filed as follows

CLAIMS:

1. A method of nucleic acid extraction from nucleic acid containing biochemical material avoiding the use of guanidinium salts, which method comprises the use of a biphasic system made up of (i) a water miscible organic solvent forming the first phase and (ii) an aqueous phase forming the second phase, in combination with a partitioning agent.

2. A method as claimed in Claim 1, wherein the water miscible organic solvent comprises a short chain alcohol.

3. A method as claimed in Claim 1 or Claim 2, wherein the partitioning agent is an inorganic salt.

4. A method as claimed in Claim 3, wherein the inorganic salt is selected from the group consisting of potassium phosphate, (mono-, dibasic or tribasic) magnesium sulphate, disodium carbonate or disodium sulphate or any mixture of these.

5. A method as claimed in any preceding claim, wherein the method comprises the steps of taking the biochemical material from which the nucleic acid is to be extracted and mixing it with the partitioning agent, one or both of the biochemical material and partitioning agent being an aqueous solution, and then adding the water miscible organic solvent; and agitating to ensure good mixing and leaving to stand for the two phases to settle out.

6. A method as claimed in Claim 5, wherein the method comprises the further step of physically separating the lower aqueous phase from the upper organic phase of the biphasic system.

7. A method as claimed in any preceding claim, wherein the biphasic system is subjected to centrifugation.

8. A method as claimed in claim 7, wherein the centrifugation is carried out for one to five minutes at or around 5000g.

9. A method as claimed in any preceding claim, which method further comprises the step of pre-treating the biochemical material with heat sufficient to denature proteins in the biochemical material.

10. A method as claimed in claim 9, wherein the heat treatment is carried out at a temperature of between 40 and 90°C.

11. A kit for use in the method of any preceding claim, wherein the kit comprises a water miscible organic solvent in a first vessel and partitioning agent in a second vessel and instructions for use of the two in accordance with the method of the preceding claims to set up a biphasic system for extraction of nucleic acid from nucleic acid containing biochemical material.

The Patent Office

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INVESTOR IN PEOPLE

Application No: GB 9819951.6 **Examiner:** Dr J Houlihan
Claims searched: 1-11 **Date of search:** 30 December 1998

Patents Act 1977
Search Report under Section 17

Databases searched:

UK Patent Office collections, including GB, EP, WO & US patent specifications, in:

UK Cl (Ed.P): C3H (HB3)

Int Cl (Ed.6): C07H 21/00, C12N 15/10

Other: ONLINE: WPI, EPODOC, DIALOG/BIOTECH

Documents considered to be relevant:

Category	Identity of document and relevant passage	Relevant to claims
X	Biotechnology Techniques **Vol.8** (7) 1994. Kimura K & Koabyashi H *"Yeast RNA partition accompanying adsorption in salt/PEG two-phase system"* pages 473-478	1, 3-7 & 11
X	BioTechniques Vol. 11 (1) 1991. *"Purification of plasmid and high molecular mass DNA using PEG-salt two-phase extraction"* pages 18-24 (see Table 1, P1-P3)	1, 3-8 & 11
X	Appl. Biochem. & Biotech. **Vol. 26** (3) 1990. Sturesson S *et.al.* *"Partition of macromolecules and cell particles in aqueous two-phase systems based on hydroxypropyl starch and poly(ethylene glycol)"* pages 281-295, especially page 286 *et.seq.*	1, 3-7 & 11

X	Document indicating lack of novelty or inventive step	A	Document indicating technological background and/or state of the art.
Y	Document indicating lack of inventive step if combined with one or more other documents of same category.	P	Document published on or after the declared priority date but before the filing date of this invention.
&	Member of the same patent family	E	Patent document published on or after, but with priority date earlier than, the filing date of this application.